Composition Studies on Tobacco XL

arge Scale Fractionation of the Neutrals of Cigarette Smoke Condensate Using Adsorption Chromatography and Solvent Partitioning*

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INTRODUCTION

Recent studies described the large scale fractionation of cigarette smoke condensate and the biological activity of the fractions, using the mouse back painting technique (1, 9). Indications of activity were obtained in a neutral subfraction which should have contained essentially no polynuclear aromatic hydrocarbons (PAH). These findings provided the first published evidence of the probable presence of nonpolynuclear compounds having such activity in the neutral fraction. To obtain stronger evidence a method was required to separate benzo[a]pyrene (BAP) and related PAH from other constituents in a highly effective manner. Recently, such a method was developed involving column chromatography of the neutrals on silicic acid followed by solvent partitioning of selected eluates (7). Using this method, enrichment of BAP from the 0.5—2.0 ppm of condensate (11) to about 105 ppm is possible. Other workers have used silicic acid chromatography to separate the total neutrals or neutral subfractions of smoke condensate for use in biological studies (2, 7, 11). With one exception (11), these fractionations have employed limited elution schemes that have not fully exploited the potentialities of adsorption chromatography. In the excepted study, an extensive eluting scheme was employed but limited biological tests were performed on the fractions.

The present study describes the large scale fractionation of the neutrals of smoke condensate by column chromatography and solvent partitioning, the levels of BAP in certain fractions and a statistical analysis of the variability of the yields of all fractions. The biological activity of the fractions will be reported elsewhere.

MATERIALS AND METHODS

Cigarette smoke condensate (CSC): This material was prepared in 1.0 kg batches at the Roswell Park Memorial Institute and shipped to Philadelphia under the conditions described previously (9). The total neutral fraction was isolated from the CSC by the solvent partition methods detailed earlier (9). A portion (41.2 %) of the total neutral fraction from each kg was removed for control purposes and the remainder was employed in the fractionation procedure.

Solvents: Petroleum ether was either nanograde or c. p.; in the latter case, the solvent was redistilled from glass. Dimethyl sulfoxide and benzene were spectral grade and used without redistillation. Methanol was nanograde and employed as received. The purification of all other solvents was described previously (9). In all solvent partitioning steps, the solvent pairs were preequilibrated before use. All fractions except those containing methanol and water were dried over magnesium sulfate or sodium sulfate; all fractions were evaporated *in vacuo* to residues at 30°—35° C.

Preparation of silicic acid columns: Silicic acid (Mallinckrodt*, 100 mesh, analytical grade) was suspended in methanol and the mixture was decanted after settling to remove the fines. This procedure was repeated three times. The moist adsorbent was then transferred to a Buchner funnel and washed with methanol until the filtrate was colorless. The adsorbent was dried on the filter and activated at 150° C for 17 hours before use. Silicic acid columns (60 mm i. d.) were prepared by moist packing using slurries of the adsorbent in petroleum ether. The sample: adsorbent ratio ($^{w}/_{w}$) was 64:1000. Since the weights of neutrals varied from batch to batch, the total weight of adsorbent was changed in each instance to maintain this ratio. After all adsorbent had been added to the columns, more petroleum ether was added and permitted to flow under pressure through the columns until the volume of adsorbent became constant. In this and subsequent eluting operations, nitrogen was employed at 10 psi to give a flow rate of 1.2 liter per hr at 25° C.

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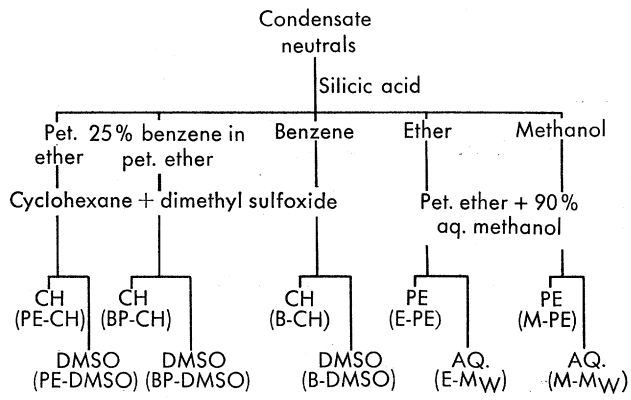
* Mention of specific commercial products does not constitute endorsement by the U. S. Department of Agriculture.

Figure 1 Fractionation of neutrals by silicic acid chromatography and solvent partitioning



Chromatography of neutrals: The sample (58.8% of the total neutrals from 1.0 kg CSC) was dissolved in a small amount (usually $\leq$ 200 ml) of petroleum ether and added to the columns. Traces of an insoluble residue usually remained in the beaker after transfer; this residue was dissolved in the minimum amount of 5% benzene in petroleum ether and added to the columns before development was initiated. Elution was then performed with solvents of increasing polarity as shown in Figure 1. The volumes of eluting solvents varied with the yields of total neutrals in successive batches of CSC. The following average volumes (liters) of eluting solvents ($\pm$ 1 standard deviation) were used in 10 such separations: petroleum ether (PE), 18.9 ± 2.7; 25% benzene in petroleum ether ($^{v}/_{v}$) (BP), 22.8 ± 3.2; benzene (B), 25.8 ± 5.4; ether (E), 21.8 ± 6.4; and methanol (M), 11.4 ± 3.0. In two cases, columns were protected from the fluorescent lighting in the laboratory during development; in the remaining instances no special precautions were taken to exclude this light. In no case were samples exposed to direct sunlight. All eluates were evaporated to residues before solvent partitioning.

Solvent partitioning of PE, BP and B eluates: The residues from these eluates were similarly treated. In each case, the residue was dissolved in 375 ml cyclohexane (CH) and the latter was extracted with 75 ml portions of dimethyl sulfoxide (DMSO) until the polar solvent removed only traces of color. Usually 5 extractions with DMSO were sufficient. The combined DMSO was cross-extracted with CH and the CH and DMSO extracts were pooled independently. To remove traces of DMSO, the combined CH extracts were washed with 15 ml of water before drying over magnesium sulfate. The pooled DMSO-extracts were cooled to 20° C during addition of an equal volume of water containing 0.33% sodium chloride to reduce emulsion formation. The 15 ml water wash of the CH was added at this step. The aqueous DMSO solution was then extracted 5 times each with alternate portions (75 ml each) of PE and E. All PE and E extracts were mixed and the combined solution was washed with 15 ml of water and dried over magnesium sulfate. The DMSO-solubles from the eluates were designated PE-DMSO, BP-DMSO and B-DMSO, respectively. The corresponding CH-solubles were designated PE-CH, BP-CH and B-CH, respectively.

Solvent partitioning of the E eluate: After removal of the eluting solvent, the residue was dissolved by successive additions of 75 ml portions of PE and 90% aqueous M. Usually, 375 ml of each solvent was required. The solvents were mixed in a separatory funnel and then separated. Each layer was shaken with the other solvent of the pair in 75 ml portions until color removal was negligible. All corresponding layers were pooled, giving total volumes of about 1500 ml for each layer. The PE extract was dried over magnesium sulfate, giving E-PE. The aqueous ME extract was designated E-M_W.

Solvent partitioning of the ME eluate: The residue from this eluate was partitioned by the same method described for the E eluate giving PE-soluble and aqueous M-soluble fractions. However, some of the residue from the eluate was insoluble in both solvents. This insoluble material was dissolved in acetone and the solution was combined with the 90% aqueous M-solubles during subsequent evaporation. The PE-soluble and aqueous M-soluble fractions were designated M-PE and M-M_W, respectively.

Reconstituted fraction: After filtration of the drying agent (when employed), 40% of each of the above extracts was pooled to provide a reconstituted fraction. The remainder of each of the solutions and the reconstituted fraction were evaporated independently to a constant weight giving a total of 10 fractions and one reconstituted sample. These 11 samples and the control of unfractionated neutrals were sealed independently under nitrogen in glass-stoppered flasks and shipped in Dry Ice for biological testing.

Removal of strongly adsorbed material: As indicated below, complete removal of the added neutrals cannot be achieved on chromatographic columns. In attempts to remove the strongly adsorbed material, one-quarter of the adsorbent was removed from the top of each of three columns of silicic acid used to fractionate the neutrals as described above. The aliquots were pooled, pyridine (3.75 liter) was added and the mixture was slurried thoroughly. The mixture was filtered and the adsorbent was again slurried with pyridine and filtered. The extracted adsorbent was then slurried twice with acetic acid (total vol, 5.5 liter) using the same method. In another procedure, a portion (40 g) of the adsorbent after elution with methanol was extracted continuously with acetone (250 ml) for 120 hrs followed by pyridine (300 ml) for 97 hrs. The solvent was removed from all extracts by evaporation *in vacuo* and the residues were packed and shipped as described above. Reflectance measurements were made on the adsorbent at various steps in the procedure using a Photoelectric Reflection Meter Model 610 (Photovolt Corp.) with magnesium oxide as a standard.

Table 2 Variability in yields of neutral fractions

Fraction Name	No.	Range[a] Mean ± 95% C. I.	V	S.D.	Coefficient of variation	Gm. per kg CSC
PE-DMSO	18	0.8 ± 0.3	0.15	0.39	49	2.3
M-PE	25	1.2 ± 0.2	0.27	0.52	43	3.4
BP-DMSO	20	2.3 ± 0.7	0.82	0.90	40	6.5
E-PE	23	16.1 ± 3.2	20.43	4.52	28	45.6
M-M_W	26	5.2 ± 1.0	2.13	1.45	28	14.7
B-CH	21	22.4 ± 3.1	18.84	4.34	19	63.4
B-DMSO	22	5.1 ± 0.8	0.93	0.96	19	14.4
BP-CH	19	9.5 ± 1.3	3.32	1.81	19	26.9
PE-CH	17	12.5 ± 1.6	5.23	2.27	18	35.4
E-M_W	24	20.2 ± 2.7	13.96	3.73	18	57.2
Reconstituted neutrals	16	63.5 ± 4.0	31.18	5.58	9	270.2
Total	16—26	158.0 ± 12.9	323.90	18.00	11	268.6

[a] Mean, $\Sigma X/10$; range (95% confidence interval), $\bar{X} \pm t_{.025} S_{\bar{X}}$; V, $\Sigma (X_i - \bar{X})^2/9$; S.D., standard deviation, $\sqrt{V}$; coefficient of variation, 100 S.D./$\bar{X}$. See Table 1 for abbreviations.

from the mean weight of F 16 (factor 0.235) and from the sum of the means of all fractions (factor 0.588).

The means and standard sampling errors in the last column of Table 1 show that variability was in general greater the smaller the mean. This is shown more clearly by Table 2 which shows that the coefficient of variation is greatest (40—49%) for means less than 5 g, and least (9—13%) for those over 25 g. The expected mean should, however, lie within the ranges shown in Table 2 for the 95% confidence interval. As previously noted, the variability in yields from different batches of CSC may be reflections of actual differences in composition as well as experimental error. Significant variations in the BAP content of successive batches of CSC were observed *(vide infra)*.

Recovery of the material placed on the columns (sum of the ten fractions plus the reconstituted neutrals) averaged 102%, with a range of 94—109 in the ten runs. As shown in Table 2, the high variability in yields of individual fractions tended to even out in the reconstituted CSC and in the sum of all fractions.

Table 3 shows the levels of BAP in the neutrals before fractionation, the reconstituted neutrals and certain neutral fractions from 4 batches of CSC. Only one of the CH-soluble fractions was analyzed since the partition coefficients of most PAH in hydrocarbon-DMSO systems are strongly in favor of DMSO (3) and previous work had shown that essentially no BAP occurs in the CH-soluble substances (8). The excitation and fluorescence spectra of the BAP isolated from BP-DMSO, the reconstituted neutrals and the unfractionated neutrals were identical with those of the authentic hydrocarbon and had no evidence of fluorescent impurities. The spectra of the prospective BAP-containing material from the other fractions were poor and considerable fluorescent interference was noted using the required limit of sensitivity of the spectrophotofluorometer. The levels shown in Table 3 for the latter fractions are the maximum concentrations possible; in all probability, BAP was either absent or present in much lower concentrations therein. For BP-DMSO, the reconstituted neutrals and the unfractionated neutrals the coefficient of variability for BAP was 5%.

The data in Table 3 show that essentially all of the BAP was found in BP-DMSO, as expected. These results confirm previous findings in which more than 96% of the BAP was found in this fraction. Based on the known chromatographic behavior of other biologically important PAH on deactivated silicic acid (5), it is highly probable that no PAH occur in E-M_W and

Table 3 Benzo[a]pyrene levels in successive lots of cigarette smoke condensate and fractions therefrom

Fraction	Fraction no.	Run no. 6	7	8	9
Neutrals before fractionation	–	0.78[a]	0.72	0.56	0.87
Reconstituted neutrals	16	0.78	0.66	0.60	0.95
Neutral fractions					
PE-DMSO	18	⩽0.05	⩽0.03	⩽0.04	⩽0.01
BP-CH	19	–	–	–	⩽0.03
BP-DMSO	20	0.74	0.64	0.55	0.84
B-DMSO	22	⩽0.05	⩽0.04	⩽0.04	⩽0.01
M-M_W	26	⩽0.03	⩽0.03	⩽0.01	⩽0.01

[a] Expressed as ppm of original CSC

BAP analyses: Analyses were performed on acetone solutions of the total neutrals, the reconstituted neutrals and selected neutral fractions that were prepared at 60% equivalent concentration for bioassay. The analytical procedures were generally similar to those employed by other workers in BAP analyses (11). To correct for losses, uniformly labelled ^{3}H-BAP (approximately 5000 dpm) was added to 5 ml of the acetone solutions in each case before analysis. The preliminary isolation steps depended on the fraction being analyzed. The total neutrals (774 mg) and the reconstituted fraction (820 mg) were evaporated to residues, the residues were dissolved in heptane and the solutions were added to activated silicic acid columns (220 mm × 13 mm). The columns were eluted successively with 25 ml of heptane and 85 ml of heptane-benzene (1:1 v/v); the latter eluates contained the BAP. For fraction BP-CH, 10 ml of acetone solution (182 mg) were evaporated to a residue which was partitioned between heptane (20 ml) and DMSO (3 × 10 ml). The pooled DMSO-extractables were treated with 30 ml of water and extracted 4 times with 10 ml of petroleum ether. The semipurified fractions obtained by these techniques together with fractions PE-DMSO, BP-DMSO, B-DMSO and M-M_W were separated further by a sequence of thin layer chromatographic fractionations on activated silicic acid, acetylated cellulose and silica gel (Distillation Products Industries) using heptane-benzene (85:15 v/v), methanol-ether-water (4:4:1 v/v) and heptane-benzene (85:15 v/v) as developing solvents, respectively. The bands corresponding to reference BAP on the final chromatograms were eluted with 3 ml benzene and the excitation and emission spectra were examined. Quantitative measurements were made at 410 and 385 mμ in the fluorescence and excitation spectra, respectively. Radioactivity in 2 ml of the isolated BAP solutions was determined using the liquid scintillation counting technique with a toluene solution of PPO (2,5-diphenyloxazole) and POPOP (2,2'-p-phenylene*bis*[5-phenyloxazole]) as the scintillators. The appropriate corrections for the recovered BAP were made.

RESULTS AND DISCUSSION

The weights obtained in the 10 separations are recorded in Table 1. For easy and positive identification during bioassay the fractions submitted for test were numbered consecutively with those (Fractions 1—14) reported in previous papers (1, 9). The composition of fraction 15 (control neutrals) is identical with that of the total neutrals before fractionation (line 1) and with that of the neutrals placed on the columns (line 3). If there were no losses or other changes produced by the fractionation procedure, the composition of fraction 16 (reconstituted neutrals) would be identical with that of fraction 15. Since the reconstituted-neutrals fraction (F 16) was obtained by combining 40% of each of the column fractions (F 17 — 26), which in turn represented 58.8% of the total neutrals, each reconstituted fraction has a theoretical weight corresponding to approximately 23.5% of the total neutrals (line 1) obtained in that run. By the same reasoning, the sum of the weights of the column fractions (17—26) should equal 35.3% of this same total neutrals weight (mean, 261.9 g). In Table 2, last column, this factor (0.353) has been used to calculate the yield of each fraction in grams that would have been obtained if the entire kilogram of CSC had been fractionated and if none had been removed after fractionation. The sum of these calculated weights is 269.8 g, agreeing closely enough with the values 270.2 g and 268.6 g calculated to the same basis

Table 1 Yields of cigarette smoke condensate fractions

Fraction[b]	No.	Run no. 1	2	3	4	5	6	7	8	9	10	Average + S.E.[b]
Total neutrals (N)	—	282.0[a]	296.1	271.8	227.2	250.0	242.4	242.2	249.0	329.0	229.2	261.9 ± 10.28
Control N (41.2%)	15	113.5	123.0	111.1	92.5	101.3	99.5	99.2	98.1	136.8	93.2	106.8 ± 4.5
N before fractionation (58.8%)	—	168.5	173.1	160.7	134.7	148,7	143.0	143.0	150.9	192.2	135.9	155.1 ± 5.7
Reconstituted N	16	68.5	66.5	66.8	55.0	62.0	63.6	64.9	62.3	71.7	53.7	63.5 ± 1.76
PE-CH	17	14.8	11.3	15.7	10.3	13.9	11.1	11.7	10.7	15.6	9.7	12.5 ± 0.72
PE-DMSO	18	1.0	0.9	0.7	0.7	0.7	1.7	0.4	0.6	0.3	0.9	0.8 ± 0.12
BP-CH	19	12.5	10.9	9.9	7.4	9.0	9.8	9.4	8.8	10.9	6.1	9.5 ± 0.8
BP-DMSO	20	4.0	2.4	3.1	1.5	1.8	3.1	1.4	1.3	1.6	2.4	2.3 ± 0.3
B-CH	21	22.1	21.5	22.6	19.9	21.3	20.0	22.4	20.5	34.4	19.6	22.4 ± 1.4
B-DMSO	22	6.5	6.1	6.1	5.6	5.1	5.2	3.4	4.7	4.4	4.3	5.1 ± 0.34
E-PE	23	16.6	20.6	14.5	10.8	9.4	15.4	16.5	22.5	22.1	12.9	16.1 ± 1.4
E-M_W	24	23.9	24.2	23.5	17.5	25.2	18.6	17.0	14.2	19.6	18.3	20.2 ± 1.2
M-PE	25	1.2	0.6	1.6	0.6	1.2	0.4	2.0	1.2	1.2	1.7	1.2 ± 0.16
M-M_W	26	5.4	4.3	4.4	6.1	6.7	1.7	6.1	6.4	5.2	5.5	5.2 ± 0.46

[a] gm from 1000 g CSC.
[b] PE, petroleum ether; CH, cyclohexane; DMSO, dimethyl sulfoxide; BP, 25% benzene — 75% PE; B, benzene; E, ether; M_W, 90% methanol — 10% H_2O. S. E. = standard error of the mean, $\sqrt{V/10}$; $V = \Sigma (X_i - \bar{X})^2/9$. 40% of the fractions 17–26 were pooled to give fraction 16. The values for 17–26 represent the yields obtained from the remaining 60%.

M-M_W and only trace amounts may be in B-DMSO using activated silicic acid as the adsorbent. The reconstituted neutrals contain essentially the same BAP level as the neutrals before fractionation indicating no gross loss of this hydrocarbon during separation. Since only batches 6 and 7 were separated using special precautions to exclude laboratory light, there was no substantial photodecomposition of BAP during the laboratory manipulations.

The weights in Tables 1 and 2 indicate that essentially all of the neutrals were eluted from the columns at the end of the separations. However, all of the columns had a light tan appearance at this time, showing that some material was retained. This pattern is invariably observed in such chromatographic separations of the neutrals of CSC. Other workers have noted that variable amounts (0.8 — 14 %) of neutrals from smoke condensate are retained on silicic acid columns after elution with methanol (4, 10, 11). Under such circumstances, total eluted weights greater than 100 % can still be obtained and are usually explained by the failure to remove the final traces of solvents from eluted fractions during evaporation.

To study this strongly adsorbed material remaining after elution with methanol, an attempt was made to remove the substances with other solvents. Pyridine and acetic acid eluted some colored material when the adsorbent was slurried with the solvents at room temperature. Significant amounts of color were also removed by continuous extraction with acetone followed by pyridine. Using reflectance as a measure of extraction efficiency and magnesium oxide as a standard (100 % reflectance), the following values were obtained for the adsorbent in these various procedures: before chromatography, 95; after methanol elution, 80; after slurrying with pyridine and acetic acid, 83; and after continuous extraction with acetone and pyridine, 89. Acetone, acetic acid and pyridine removed the equivalent of 0.79, 1.02 and 1.62 gm per kg of condensate, respectively, in all of these operations. These findings indicate that the strongly adsorbed material represents only a small part of the total neutrals of condensate.

Some work was done concurrently on the chemical composition of the BAP-containing fraction, BP-DMSO. At least 480 gas chromatographic peaks can be observed therein and about 75 % of these peaks are smaller in size than the BAP peak. The fraction contains a significant amount of high molecular weight components and/or heat labile compounds that do not elute when gas chromatographic separations at high temperature are employed. The major components isolated to date are PAH, aromatic amines and chlorinated hydrocarbons arising from pesticidal residues on the tobacco (6).

SUMMARY

Ten successive 1 kg samples of cigarette smoke condensate were prepared and the neutrals were removed by solvent partitioning of each kg. After removal of a control sample (41.2 %), the remainder of the neutrals were separated by adsorption chromatography on silicic acid followed by partitioning of the eluates between polar and nonpolar solvents, yielding a total of 10 fractions for biological study. A range of recoveries from 94—109 % of the neutrals was obtained in the ten successive runs; the overall average recovery was 102 %. Small amounts of strongly adsorbed material not eluted from the adsorption columns by the technic could be removed in part by other procedures. Using this method, benzo[a]pyrene was concentrated to a high degree in one fraction, thus permitting the detection of biologically active nonpolynuclear agents in the other fractions on bioassay. Exclusion of light during separation did not alter significantly the benzo[a]pyrene levels obtained in the polynuclear-enriched fraction.

ZUSAMMENFASSUNG

Nacheinander wurden zehn 1-kg-Proben Cigarettenrauchkondensat gewonnen und die Neutralstoffe aus jedem kg durch Lösungsmittelverteilung abgetrennt. Nach Abnehmen einer Vergleichsprobe (41,2 %) wurde der verbleibende Rest für biologische Untersuchungen durch Adsorptionschromatographie an Silicagel gefolgt von der Verteilung der Eluate zwischen polaren und unpolaren Lösungsmitteln in 10 Fraktionen aufgetrennt. Mit einer Range von 94 — 109 % wurden die Neutralstoffe bei den zehn aufeinanderfolgenden Gängen zurückerhalten; die durchschnittliche Gesamtausbeute betrug 102 %. Kleine Anteile von fest adsorbiertem Material, das bei dieser Technik nicht von der Adsorptionssäule eluiert wurde, konnten teilweise durch andere Methoden zurückerhalten werden. Bei Anwendung dieser Methode wurde das Benzo(a)pyren in hohem Grade in einer Fraktion konzentriert; dadurch wurde die Bestimmung der biologischen Aktivität nichtpolynuklearer Agenzien in den anderen Fraktionen im biologischen Experiment ermöglicht. Ausschluß von Licht bei der Auftrennung veränderte nicht signifikant den Benzo(a)-pyrengehalt in der Fraktion, die die polynuklearen Verbindungen angereichert enthielt.

RÉSUMÉ

On a préparé 10 échantillons successifs de 1 kg de condensat de fumée de cigarette et on en a extrait les composants neutres en soumettant chaque échantillon à partage entre solvants. Après avoir prélevé un échantillon-témoin (41,2 %), le reste des composants neutres a été fractionné par chromatographie d'adsorption sur acide silicique puis les éluats ont été soumis à partage entre solvants polaires et non-polaires; on a ainsi obtenu 10 fractions pour bioessai. Les 10 séries successives ont donné une gamme de récupération des composants neutres s'étageant entre 94 et 109 %; la moyenne générale étant 102 %. De petites quantités d'une substance fortement adsorbée qui n'avait pu être éluée par la technique habituelle ont pu être récupérées

par d'autres procédés. En utilisant cette technique, le benzo(a)pyrène a été fortement concentré dans une seule fraction, permettant ainsi la détection, par bioessai, d'agents actifs non polynucléaires dans les autres fractions. En procédant à la séparation à l'abri de la lumière on n'a pas modifié significativement les taux de benzo(a)pyrène obtenus dans la fraction enrichie en ce corps.

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